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Review

The Evaluation of Methods for Detection of *Bacillus Anthracis* Spores in Artificially Contaminated Soil Samples

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Abstract

The nested PCR has been used to evaluate the usefulness and efficiency of different *Bacillus anthracis* spore isolation methods in contaminated soil samples. The best results were obtained using two methods described by Beyer et al. [1] and Cheun et al. [9]. Outer and inner pairs of primers were designed from the protective antigen gene of plasmid pXO1 as well as from genes B and C of the capsule region of the plasmid pXO2. The influence of soil types on obtained results was also studied. The type of soil samples did not affect the nested PCR results. Furthermore, the sensitivity of nested PCR and PCR – ELISA was also examined.

Keywords: *Bacillus anthracis* spores, nested PCR, PCR – ELISA

Introduction

The process of isolation and identification of *Bacillus anthracis* spores from environmental samples is more difficult and complicated in comparison to the process performed on clinical samples [1-7]. The difficulty is related to the presence of organic and inorganic compounds in soil. The compounds interfere and inhibit the particular steps of the diagnostic process (mainly DNA isolation). Furthermore, the presence of various bacterial floras in soil samples (mainly from *Bacillus* species) hampers the *Bacillus anthracis* identification process [1-3, 6]. Recently, new methods of isolation of *Bacillus anthracis* from soil samples have been introduced. The methods are based on the application of the two-step culture on the non-selective enrichment medium for bacterial growth [2, 8, 9]. The aim of this work was to evaluate selected isolation and identification methods for detection of *Bacillus anthracis* spores in artificially contaminated soil samples and to verify the influence of the soil types on the obtained results.

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Experimental Procedures

The Bacterial Strains

The *Bacillus anthracis 34F*$_2$ (obtained from the commercial anthrax spore vaccine, Anthraphyl – Sanofi) and *Bacillus anthracis 211* isolated from cow spleen (obtained from Veterinary Hygiene Institute in Łomża). The soil samples were collected from sandy (SS – sandy samples), forest (FS) and wetland (WS) areas. The 100g of samples were artificially contaminated by 10^8–10^0 CFU/ml of *Bacillus anthracis 34F*$_2$ spores and tested for the presence of the *pag* gene by nested PCR. Three *Bacillus anthracis* isolation methods from soil samples were used in

this study. The two methods were performed according to the procedure described by Beyer et al. [1] and Cheun et al. [9]. The third – the thermal method also used in research was performed according to the following procedure. Hundred milligrams of soil sample were resuspened in 1.5 ml of trypticase soy broth (TSB) and boiled for 10–15 min. In order to concentrate and remove the solid compounds present in tested material, the sample was centrifuged (11,000–25,000 rcf) and washed by distilled water.

DNA Isolation

The DNA from each strain was obtained using the Genomic DNA Prep Plus (A&A Biotechnology Poland) according to manufacturer protocol. The quality of the DNA from each strain was verified on 0.7% agarose gel containing 1 µg/µl ethidium bromide.

Standard PCR reaction contained 10 x PCR buffer (Sigma) (100 mM Tris-HCl, pH 8.3; 500 mM KCl; 11 mM $MgCl_2$; 0.1% gelatin), deoxynucleotide mix (Sigma) (2 mM dATP, 2 mM dCTP; 2 mM dGTP; 2 mM TTP); 1 mM $MgCl_2$, REDTaq DNA Polymerase (Sigma) (20 mM Tris-HCl, pH 8.0; 100 mM KCl; 0.1 mM EDTA; 1 mM DTT; 0.5% Tween 20; 0.5% igepal CA-630; inert dye; 50% glycerol 1U; 20 pmol of each primer; 2.5 µl of DNA as a template. The PCR was performed in iCycler (Bio-Rad). The primers used in PCR. The *pag* and *cap* coding regions were PCR-amplified from DNA template using the following primers (5'–3'):

PA 5: TCCTAACACTAACGAAGTCG,
PA 8: GAGGTAGAAGGATATACGGT
Cap 6: TACTGACGAGGAGCAACCGA,
Cap 103: GGCTCAGTGTAACTCCTAAT

The PCR Technique

The DNA template was denatured at 94°C for 5 min. Amplification was performed using 35 cycles, with each cycle consisting of denaturation step at 94°C for 30 sec, annealing at 58°C for 30 sec and extension at 72°C for 30 sec. The final extension was accomplished at 72°C for 7 min. The PCR was performed using the iCycler (Bio-Rad). The PCR products were electrophoresed on 2% agarose gel (Sigma) containing 1µg/ µl ethidium bromide. The TBE (Sigma) buffer (0.5x) was used for preparing the agarose gel and for electrophoresis. The PCR products were electrophoresed using Blue Marine apparatus (Serva Poland). The results were imaged on gel registration system ImageMaster® VDS (Pharmacia Biotech).

The nested PCR contained the mixture as previously described. In the second step of the reaction the 2.5µl of amplicons were used as a template. The sequence of inner pairs of primers used in the reaction (5' – 3') were:

PA 6: ACCAATATCAAAGAACGACGC,
PA 7: ATCACCAGAGGCAAGACACCC
Cap 9: ATGTATGGCAGTTCAACCCG,
Cap 102: ACCCACTCCATATACAATCC

PCR and electrophoresis were performed as described above.

The PCR – ELISA contained the mixture previously described with one exception. The deoxynucleotide mix was replaced by PCR – DIG labeling mix (Boehringer Mannheim GmbH) containing 2 mM dATP, dCTP, dGTP; 1.9 mM dTTP and 0.01 mM DIG – 11 dUTP. The digoxigenin-marked PCR products were detected by ELISA test using the Dig Detection Kit (Boehringer Mannheim GmbH). The ELISA tests were performed according to the manufacturer's protocol. The ELISA results were viewed on Ultramark ELISA reader (Bio-Rad) at 405 nm wavelength. The results were compared to positive control (human control) and negative control (*Bacillus cereus ATCC 10876*).

The PCR-ELISA sensitivity test was performed using serial dilution (10 ng/µl–10 ag/µl) of *Bacillus anthracis* plasmid DNA.

Statistical analysis was performed using "Statistica™PL" software with relevance parameter (P) on the level of P=0.05 [10].

Results

The Evaluation of the Nested PCR Method for Detection of *Bacillus anthracis* Spores in Artificially Contaminated Soil Samples in Relation to the Different Methods of Spore Isolation

There were no amplification products of the *pag* gene observed in the first round of reaction (Figs. 1, 2). In the second round, the PCR products of expected size 210 bp

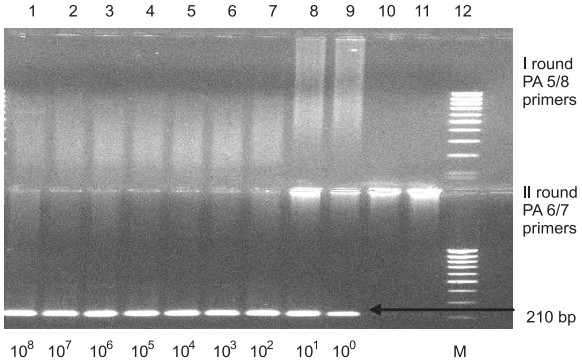

Fig. 1. The influence of Beyer's spore isolation method on nested – PCR results (FS – Forest Sample.)
The legend: Line 1–9: Tenfold serial dilution of *Bacillus anthracis* spores. Line 10: Negative control (the soil sample without *Bacillus antharcis* spores). Line 11: Negative control (the PCR mastermix control). Line 12: M – 100 bp ladder (Fermentas).

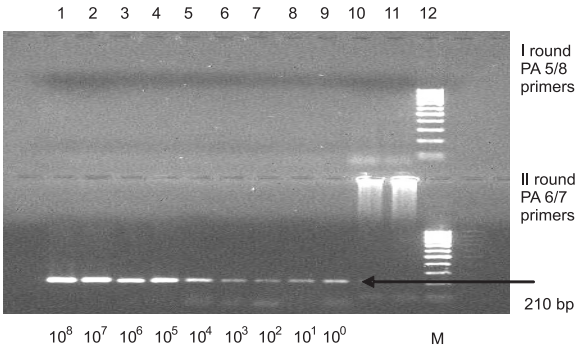

Fig. 2. The influence of Cheun's spore isolation method on nested – PCR results (FS – Forest Sample).
The legend: Line 1–9: Tenfold serial dilution of *Bacillus anthracis* spores. Line 10: The negative control (the soil sample without *Bacillus antharcis* spores). Line 11: The negative control (the PCR mastermix control). Line 12: M – 100 bp ladder (Fermentas).

(base pair) were discovered in all artificially contaminated from 10^8 to 10^0 CFU/ml soil samples. The nested PCR level of detection ranged from 1 to 10 bacterial cells.

The SS, FS, and WS soil samples were artificially contaminated by *Bacillus anthracis* $34F_2$ 10^8 to 10^0 CFU/ml spores. Spore isolation was performed according to the procedure described by Beyer et al. [2]. There were no amplification products observed in three types of soil samples in the first round of nested PCR. In the second round the PCR amplicons of expected size 210 bp were observed in all types of soil samples. The influence of soil types on obtained results were not discovered.

The Sensitivity of PCR-ELISA Method

The soil samples were artificially contaminated by *Bacillus anthracis* tenfold increasing serial concentration of spores ranged from 10^0 to 10^8 CFU/ml. The *Bacillus anthracis* isolation was performed according to the procedure described by Beyer et al. [2]. The sensitivity of PCR–ELISA method was established on the level of 10^1 to 10^2 CFU/ml (Fig. 3).

The Sensitivity of PCR–ELISA and Nested PCR Methods Comparison Tests

The tenfold of *Bacillus anthracis* plasmid DNA serial dilution (10 ng/µl – 10 ag/µl) were used. The results of test are listed on Figs. 4 and 5. In the first round of nested PCR, the sensitivity threshold ranged on the level of 10 pg DNA/µl, while in the second on the level of 1 fg/µl. The PCR-ELISA method sensitivity was established on the level of 10 fg/µl (Fig. 5).

Discussion of Results

Many researchers suggest that the *Bacillus anthracis* detection and identification process in clinical samples

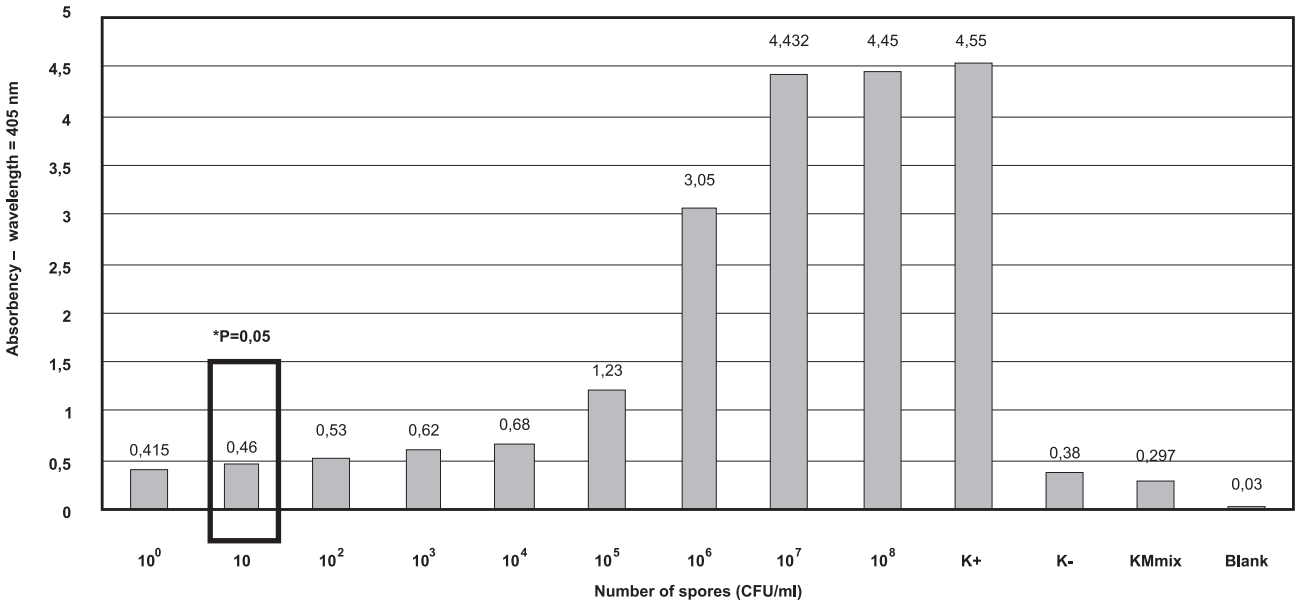

Fig. 3. The detection of *Bacillus anthracis* $34F_2$ spores in artificially contaminated soil samples using PCR-ELISA method.
The legend: **(K–)** Negative control – *Bacillus cereus ATCC 10876,* **(K+)** Positive control – the PCR amplicon after reaction with primers specific for human Tissue Plasminogen Activator (tPA), **KMmix** – The PCR mastermix control, **Blank** – The empty sample
***** The relevance of substraction results among tested parameter values was calculated using the relevance parameter on the level of $P< 0.05$.

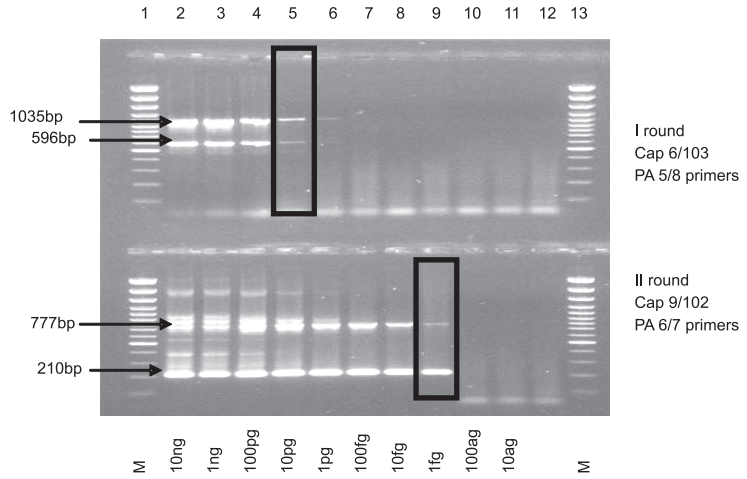

Fig. 4. The sensitivity of nested PCR assay. Line 1 and 13: M – 100 bp ladder (Fermentas).
The legend: Line 2–11: Tenfold serial dilution of *Bacillus anthracis* plasmid DNA (ng/ µl – ag/ µl). Line 12: The negative control.

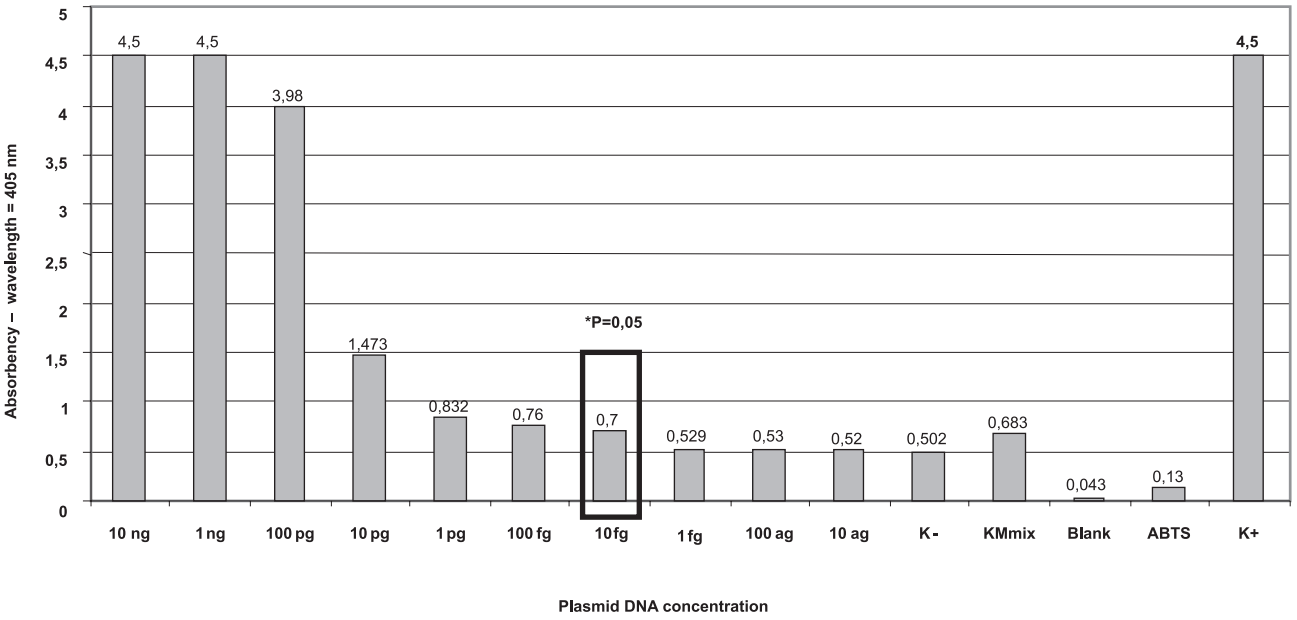

Fig. 5. The PCR – ELISA assay sensitivity to detect the *pag* gene amplicon.
The legend: **(K–)** Negative control – *Bacillus cereus ATCC 10876*, **KMmix** – The PCR mastermix control, **Blank** – The empty sample, **ABTS** – The color sample with ABTS (2.2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid)), **(K+)** – The positive control with control primers specific for human Tissue Plasminogen Activator (tPA).
The relevance of substraction results among tested parameter values was calculated by using the relevance parameter on the level of $P< 0.05$.

does not cause any problems in comparison to the environmental samples [1, 2, 11]. It's related to the presence of bacterial flora in environmental samples (mainly spore forming from *Bacillus sp.*). Furthermore, the presence of organic and inorganic compounds interferes with the *Bacillus anthracis* detection and identification process [1, 2, 11-14].

In this study, *Bacillus anthracis* spore isolation from soil samples was performed according to the methods described by Beyer et al. [1, 2], Cheun et al. [9] and a method characterized as thermal. The main difference, which distinguished the first two methods from thermal, is implementation of two steps culturing process on non-selective enrichment medium for bacterial growth. In contrast, the reported conventionally used selective agar plates (PLET – medium) are not an optimal for *Bacillus* spore recovery [15]. Furthermore, in the method described by Beyer et al. [1, 2], the vegetative cells (which contaminated the sample) bacteriocidal action was performed using a 30% solution of hydrogen perox-

ide, while in the Cheun et al. [9] method the process was carried out by 70% ethanol. Moreover, the difference between these methods is observed also in the time of two steps of culturing process. In the first case the incubation time come to the values of 18 h and 6 h in TSB, while in the second it comes to 12 h and 4 h [16]. In relation to the time factor needed to execute the first two methods, the usefulness of thermal method was also verified. The isolation of *Bacillus anthracis* spores according to this method was performed by boiling the sample in 100°C for 15 min.

All three methods were useful for DNA isolation; however the first two were more efficient in comparison to the third. Furthermore, the PCR bands visualized in gel registration system were also more readable. Probably, these facts are related to the elimination of PCR inhibition substances (during the DNA isolation process) existing in soil. According to Tebbe et al. [17], this problem is a result of the presence of PCR inhibition substances in soil e. g.: the humus substances and inorganic compounds [1-3]. Moreover, the activity of Taq polymerase can be reduced in case of DNA direct isolation from soil samples [17]. According to Cheun et al. [9], the DNA isolation based on the two-step culturing process allowed us to obtain stable PCR products and enabled the elimination of phospholipids, which may interfere with Taq polymerase. Furthermore, the two steps culturing on the non-selective enrichment medium eliminate chemical contamination, which inhibits the PCR [9].

In our studies the thermal method for DNA isolation (boiling the sample in 100°C for 15 min) was also used. Dragon et al. [15] used the temperature 65–80°C for 15 min., which allowed them to detect the spores in soil samples [4, 6, 18, 19, 20]. However Cheun et al. [9], suggested that the thermal isolation method produced the unstable PCR products and may cause false positive results [9]. Another method introduced by Yeates et al. [14] is based on using the bead beating and sodium dodecyl sulfate (SDS) for DNA isolation from soil samples. DNA is obtained by precipitation with potassium acetate, phenol, isopropanol and polyethylene glycol. DNA isolation was also performed using ultrasounds [14], but above-mentioned methods were too complicated for wider use.

The results of sensitivity tests, where the PCR, nested PCR and PCR–ELISA methods were compared, it was stated that the standard PCR was 10^4–fold less sensitive than nested PCR method. Furthermore, the PCR–ELISA test was 10^3–fold more sensitive than standard PCR method. The obtained results are comparable with results described by Beyer et al. [3]. In relation to these data, the nested PCR method should be advised for soil sample examination [1, 2].

In summary, the best results were obtained using the methods described by Beyer et al. [1] and Cheun et al. [9]. Furthermore, it was observed, that the type of artificially contaminated soil did not influence results obtained by nested PCR method.

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